Development/Plasticity/Repair

Sonic Hedgehog Has a Dual Effect on the Growth of Retinal Ganglion Axons Depending on Its Concentration

Adrianne Kolpak,* Jinhua Zhang,* and Zheng-Zheng Bao
Department of Medicine and Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

The stereotypical projection of retinal ganglion cell (RGC) axons to the optic disc has served as a good model system for studying axon guidance. By both *in vitro* and *in vivo* experiments, we show that a secreted molecule, Sonic hedgehog (Shh), may play a critical role in the process. It is expressed in a dynamic pattern in the ganglion cell layer with a relatively higher expression in the center of the retina. Through gel culture and stripe assays, we show that Shh has a dual effect on RGC axonal growth, acting as a positive factor at low concentrations and a negative factor at high concentrations. Results from time-lapse video microscopic and stripe assay experiments further suggest that the effects of Shh on axons are not likely attributable to indirect transcriptional regulation by Shh. Overexpression of Shh protein or inhibition of Shh function inside the retina resulted in a complete loss of centrally directed projection of RGC axons, suggesting that precise regulation of Shh level inside the retina is critical for the projection of RGC axons to the optic disc.

Key words: **retinal; ganglion cell; axon; optic disc; factor; guidance**

Introduction

During development of the visual system, retinal ganglion cells (RGCs) inside the retina differentiate in a wave-like manner, initiating in the center of the retina and propagating toward the periphery. Following the same order, axons at the center of the retina grow out before those at more peripheral positions (Thanos and Mey, 2001; Oster et al., 2004). Regardless of their positions, however, all RGC axons project to the optic disc at the center of the retina, in which they exit the eye to join the optic nerve. In chick, the first ganglion axons are found in the dorsal central retina at Hamburger-Hamilton (HH) stage 15 [embryonic day 2 (E2) to E3] (Goldberg and Coulombre, 1972; Kahn, 1973; Horder and Mashkas, 1982; Rager et al., 1993). By E8, most of the retinal axons have reached the optic disc except those at the very periphery of the retina.

Basal lamina, chondroitin sulfate proteoglycans, a receptor tyrosine phosphatase CRYPα, and proteins of the Ig superfamily, including L1, N-CAM (neural cell adhesion molecule), and DM-GRASP (neurolin), are implicated in the retinal axon projection toward the optic disc (Snow et al., 1991; Brittis et al., 1995; Giordano et al., 1997; Halfter and Schurer, 1998; Ott et al., 1998; Ledig et al., 1999). With the discovery of an increasing number of axon guidance cues (Tessier-Lavigne and Goodman, 1996; Flanagan and Vanderhaeghen, 1998; O'Leary and Wilkinson, 1999), the function of some of the axon guidance cues in guiding RGC axons inside the retina has been examined recently. Slit1-expressing amacrine cells provide intermediate targets for RGC axons (Jin et al., 2003). In netrin1/DCC (Deleted in Colorectal Cancer) or EphB ligand-deficient mice, defects in ganglion axon targeting were observed near the optic disc (Deiner et al., 1997; Birgbauer et al., 2000). Recently, we have shown that a zinc finger protein Zic3 is involved in patterning the retina to regulate the expression of a negative factor in a gradient high in the periphery (Zhang et al., 2004).

Sonic hedgehog (Shh), a known morphogen that plays many important roles during vertebrate development (McMahon et al., 2003), has been shown recently to directly act on axons. It is shown to act as a chemoattractant that collaborates with netrin-1 in midline axon guidance (Charron et al., 2003) and act as a negative factor to direct retinal ganglion axonal growth at the diencephalic ventral midline (Trousse et al., 2001). In both cases, the effect of Shh on axons has been shown not through repatterning of the target tissues or gross alteration of neural differentiation but through a direct effect of Shh on the growth cones of the targeted neurons.

Here, we show that Shh can act both as a positive and a negative factor on RGC axonal growth depending on its concentration. Results from time-lapse video microscopy and stripe assay suggest that the effects of Shh on RGC axons are not likely attributable to indirect transcriptional regulation by Shh. *In vivo* experiments further support that Shh may be a critical factor involved in the directed growth of the RGC axons to the optic disc.

Materials and Methods

Injection of retrovirus and cyclopamine in chick embryos. All experimental manipulations were performed on standard specific pathogen-free white Leghorn chick embryos, provided fertilized by Charles River Laboratories (North Franklin, CT). Eggs were incubated inside a moisturized 38°C incubator and staged according to Hamburger and Hamilton (1992). The replication-competent avian retroviral construct RCAS-Shh ob-

Received Dec. 3, 2004; revised Feb. 14, 2005; accepted Feb. 17, 2005.

This work was supported by grants from the Worcester Foundation for Biomedical Research and National Eye Institute. We thank Yongmei Pei for technical assistance, Mary Chau for helpful comments on this manuscript, Dr. Paul Furcinitti for help with the time-lapse imaging analysis, and Drs. Virginia Lee and Cliff Tabin for reagents.

*A.K. and J.Z. contributed equally to this work.

Correspondence should be addressed to Zheng-Zheng Bao, Department of Medicine and Cell Biology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605. E-mail: zheng.bao@umassmed.edu.

DOI:10.1523/JNEUROSCI.4938-04.2005

 0270-6474/05/253432-10$15.00/0

tained from Dr. Cliff Tabin's laboratory at Harvard Medical School (Boston, MA) has been described previously (Riddle et al., 1993). RCAS-Shh virus was prepared by transfection of a chicken fibroblast line, DF1, and concentrated by ultracentrifugation (Bao et al., 1999).

For treatment of the chicken embryos with cyclopamine, 1 mg of cyclopamine (Toronto Research Chemicals, Toronto, Ontario, Canada) was complexed with 45% 2-hydroxypropyl-β-cyclodextrin (HBC) (Sigma, St. Louis, MO) in PBS by stirring for 1–2 h at 65°C (Incardona et al., 1998). Cyclopamine-HBC was microinjected into the vitreal space of embryonic chick eyes at E3.5 or, for some experiments, into the optic vesicles at HH stage 10 (E1.5). Control embryos were injected with HBC alone.

In situ *hybridization and immunofluorescent staining. In situ* hybridization procedures on cryosections or flat-mount retinas were similar as described previously (Jin et al., 2003; Zhang et al., 2004). Chick eyes dissected at various embryonic ages were fixed in 4% paraformaldehyde in PBS overnight at 4°C and cryoprotected in 30% sucrose. The blocks were made by embedding samples in OCT (Sakura, Torrance, CA), and cryosections were prepared at 20 μm thickness on a cryostat (CM 3050S; Leica, Nussloch, Germany). Riboprobes were labeled with digoxigenin (DIG) by incorporating DIG-UTP (Roche, Basel, Switzerland) using *in vitro* transcription. Each slide was hybridized with a digoxigenin-labeled riboprobe at ~1 ng/ml in 70 μl of hybridization solution (50% formamide, 5× SSC, pH 4.5, 50 μg/ml yeast RNA, 1% SDS, and 50 μg/ml heparin) for 12–16 h at 70°C. Axons were stained with a monoclonal antibody 270.7 (generously provided by Dr. Virginia Lee, University of Pennsylvania Medical School, Philadelphia, PA). Viral infection was confirmed by immunofluorescent staining with either a monoclonal antibody 3C2 (diluted 1:5) or a polyclonal antibody p27 (diluted 1:10,000; SPAFAS, Norwich CT).

Gel culture assay. E6 chick retinas were cut into small pieces in HBSS and transferred into a 24-well plate. Bovine type I collagen (BD Biosciences, San Diego, CA) neutralized with 1 M $NaHCO_3$ and 10× DMEM was added to the explants and allowed to gel for 30–40 min at 37°C. A recombinant Shh protein containing the functional N terminus (Shh-N) (R & D Systems, Minneapolis, MN) was added at a range of concentrations from 0.15 to 2.5 μg/ml to the culture media of retinal explants and cultured for 44 h at 37°C, 5% CO_2. In some experiments, 2.5 μM cyclopamine was added with Shh-N protein in the culture medium. For the retinal coexplant experiment, retinal tissues to be tested were embedded into 2% low-melting agarose to prevent the growth of axons, modified from a previously published procedure (Richards et al., 1997). One central or one peripheral half of the retina was embedded into each agarose block. The tissue blocks were trimmed so that a very thin layer (~100 μm) of agarose remained outside of the retinal tissues. The tester retinal tissues were placed 100–300 μm away from the agarose block, and collagen was added to cover both the agarose block and the tester explants.

After 44 h, the cultures were fixed with 4% paraformaldehyde. The axons were stained with anti-neurofilament antibody 270.7, examined using an inverted microscope (Eclipse E400; Nikon, Tokyo, Japan), and photographed with a digital camera (SPOT camera; Diagnostic Instruments, Sterling Heights, MI). The axons were traced digitally with Adobe Photoshop software (Adobe Systems, San Jose, CA) and quantified by measuring pixels using the NIH Image program. All data sets were calculated from at least 10 retinal explants in three independent experiments. The average axon length was expressed as means ± SEM. Unpaired Student's *t* test was used to assess statistical significance between data sets.

Time-lapse video microscopy. E6 chick retinas were dissected and flattened onto a nitrocellulose membrane. Thin strips of retina were cut and cultured in a 35 mm coverslip dish (MatTek, Ashland, MA) coated with 20 μg/ml poly-D-lysine (Sigma) and 10 μg/ml laminin (BD Biosciences). After 20–24 h in the culture medium DMEM/F-12 (1:1) supplemented with N2 (Invitrogen, Rockville, MD), the axons were recorded by using an Olympus Optical (Tokyo, Japan) Axiovert microscope with temperature and CO_2 control, coupled to a CoolSnap HQ CCD video camera (Roper Scientific, Trenton, NJ). Time-lapse images were obtained for 1 h at 15 min intervals before the addition of 0.5 μg/ml Shh-N protein or BSA control. Time-lapse recordings were immediately resumed after the addition of proteins for an additional 3 h at 15 min intervals.

Using NIH Image software, axonal growth was measured by monitoring changes in pixel coordinates (x, y) of the leading edge of individual growth cones for all axons visible in the field over the entire 4 h filming period. Axonal growth during each time interval was determined by calculating the change in coordinates of the growth cones between consecutive specified time points using the formula $\sqrt{[(y2 - y1)^2 + (x2 - x1)^2]}$. A positive value was assigned for the growth cones that extended, and a negative value was assigned for the growth cones that retracted. Cumulative axon growth was determined by summing the growth during each time interval beginning with the initial position 1 h before protein addition. Forty-one axons from four independent experiments treated with Shh-N and 28 axons from three independent control experiments were measured, and growth was expressed as the average ± SEM cumulative axon length. Statistical significance was calculated using unpaired Student's *t* test.

Stripe assay. Stripe assays were performed essentially as described previously (Vielmetter et al., 1990). Glass coverslips were coated with 20 μg/ml poly-D-lysine (Sigma) and then mounted on the silicone matrix (Max-Planck-Institute, Tübingen, Germany). For control experiments, 0.1% BSA, 10 μg/ml laminin (BD Biosciences), and 1:100 cyanine 3 (Cy3)-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) were added to the inlet channel and incubated at either room temperature or 37°C for 2 h to produce the first stripes. The channels were then washed with PBS, the coverslip was removed, and the second stripe, which contained 0.1% BSA and 10 μg/ml laminin, was added at room temperature for 2 h. The coated coverslips were then washed twice with PBS. For experiments investigating the role of Shh in axon guidance, 0.5 μg/ml and 2.5 or 4.0 μg/ml purified Shh-N was substituted for 0.1% BSA in the first stripes to test low and high concentrations of Shh, respectively.

E6 retinas were dissected and flat mounted on nitrocellulose, cut into thin strips, and placed perpendicular to the direction of the stripes. Retinal explants were cultured in F-12 media containing 0.4% methyl cellulose (Sigma) and penicillin/streptomycin at 37°C, 5% CO_2, 100% relative humidity for 48 h and then fixed with 4% paraformaldehyde. Axons were stained with an anti-neurofilament antibody (270.7) followed by an FITC-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch).

Fluorescent images of the axons and stripes were acquired and merged using Spot Advanced software (Molecular Devices, Sunnyvale, CA), and results were quantified by first tracing axons using Adobe Photoshop software. The number of pixels on the first stripe (containing the fluorescent antibody) and second stripe for each region that covered five total stripes were quantified using NIH Image software and normalized for the width of the stripes. Five regions from three independent control and Shh-treated stripe assays were quantified, and results are expressed as the ratio of axonal growth on the second stripe versus axonal growth on the first stripe ±SEM. Statistical significance was calculated using the unpaired Student's *t* test.

Results

A secreted factor was found in the retina to promote the growth of RGC axons

To determine whether positive or negative factors are present endogenously at different regions of the retina to influence the growth of the RGC axons, we performed coculture gel culture experiments. Chick retinas were dissected at E5, E6, and E8, and the central and peripheral halves of the retinas were embedded separately into 2% low-melting agarose blocks (Fig. 1*F*). The agarose blocks were trimmed so that only a very thin layer of agarose (~100 μm) remained outside of the retinal tissues. The small "tester" retinal explants were placed ~100–300 μm away from the agarose blocks. After 44 h, although a normal number of axons grew out from the tester explants, few axons were observed extending from the embedded retinal tissues (Fig. 1*C*), probably because of the presence of the agarose block. This shows that a

large number of axons would not extend from the embedded tissues to interfere with the axons from the tester explants, and the coculture gel culture can be used to test secreted activity from the embedded tissues.

We measured the length of the RGC axons in different coculture conditions and compared the data with the "blank" control in which empty agarose blocks lacking any retinal tissues were cocultured with the tester explants (Fig. 1). Unpaired Student's *t* test was used for statistical assessment of the pairwise comparison. Compared with the blank controls (mean ± SEM of the axon length, 236 ± 17.8 μm) (Fig. 1*A*), the axons from the tester explants cocultured with the tissues from E5 central retina were significantly longer (363.0 ± 46.9 μm; $p < 0.001$; Student's *t* test) (Fig. 1*B*), suggesting that the E5 central retina secretes a positive factor that affects axonal growth. In contrast, the length of axons cocultured with the E5 peripheral retina was not significantly different from that of the control (224.2 ± 40.2 μm; $p > 0.5$) (Fig. 1*C*), suggesting that the E5 peripheral retina does not have any detectable secreted activity. Similarly, a positive activity was detected in the E6 central retina (axonal length, 372.0 ± 45.7 μm; $p < 0.001$) but not in the peripheral retina (axonal length, 236 ± 36.9 μm; $p > 0.5$). At E8, however, the positive activity was only found in the retinal periphery (axonal length, 622 ± 71.0 μm; $p < 0.001$) (Fig. 1*E*) and not in the center (axonal length, 290 ± 57.0 μm; $p > 0.1$) (Fig. 1*D*). These results suggest that there is a positive activity secreted by the retinal tissues that stimulates the growth of RGC axons. Because the differentiation of retinal ganglion cells proceeds in a wave-like manner from retinal center to periphery, the activity appears to move with the progression of axonogenesis from retinal center to periphery.

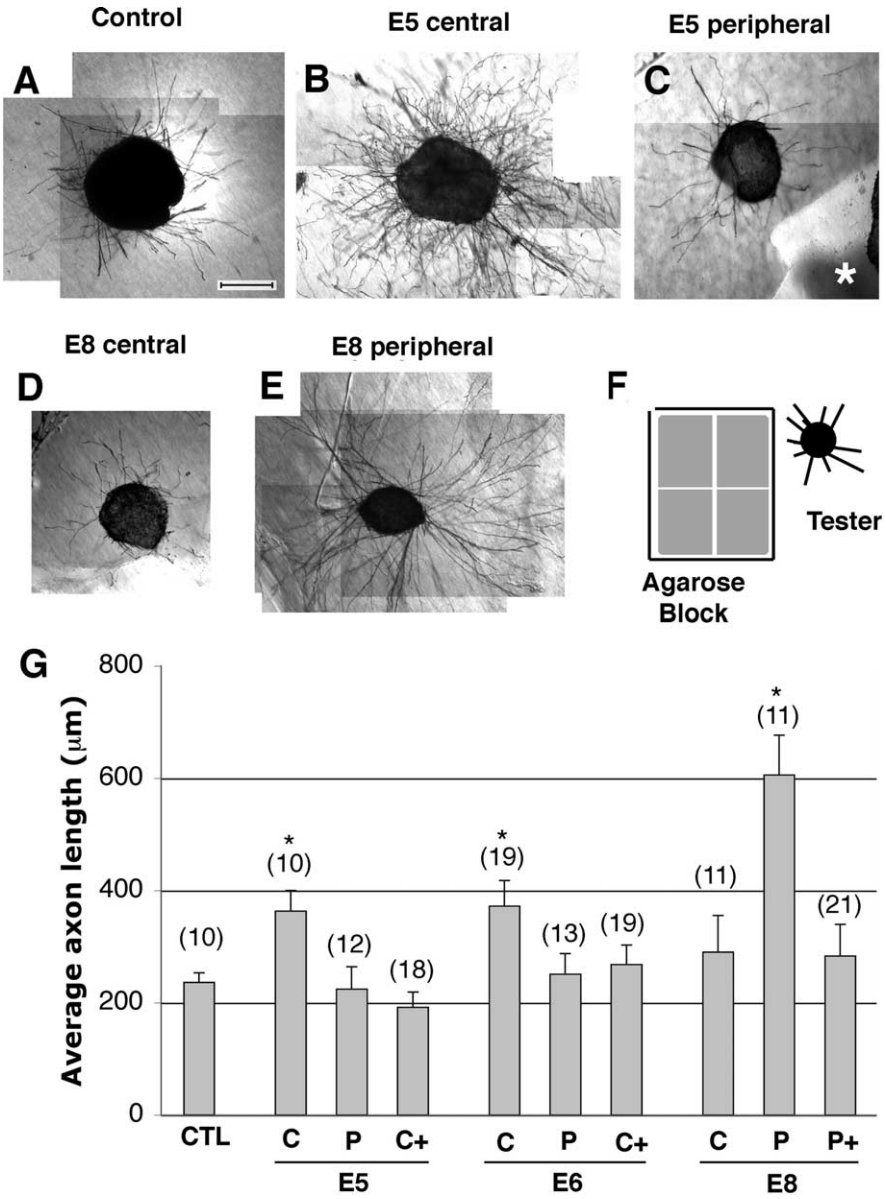

Figure 1. An endogenous positive factor was detected inside the retina. Central and peripheral halves of E5, E6, and E8 retinas were embedded separately into 2% low-melting agarose blocks and cocultured with tester retinal explants, as schematically represented in ***F***. After 44 h, axons of the tester explants in different coculture conditions were stained and photographed. Some examples are shown: ***A***, with control empty agarose block; ***B***, with E5 central; ***C***, with E5 peripheral (* marks retinal tissue embedded in 2% agarose); ***D***, with E8 central; ***E***, with E8 peripheral retina. The data were quantified and are shown in ***G***. C, Center; P, periphery; +, with cyclopamine. Note that a positive activity for RGC axonal growth was detected in E5, E6 central, and E8 peripheral halves of the retinas but not in the other halves of the retina. Furthermore, this positive activity could be abolished by inclusion of cyclopamine. The number of samples assayed was shown in parentheses, and statistical significance compared with the control (CTL) is indicated (*$p < 0.001$). Scale bar, 200 μm.

Shh mRNA is expressed in a dynamic pattern in the developing retina

To identify the factor that promotes the growth of the RGC axons, we searched for the gene that is expressed high in the center and low in the periphery of the retina at E5–E6, by testing candidate molecules. By *in situ* hybridization on flat-mount and cross sections of chick retinas, we found that *Shh* mRNA is expressed in dynamic patterns correlating with the progression of retinal ganglion cell differentiation and axonogenesis. At early stages such as E4.5, E5.5, and E6 (Fig. 2), the expression of *Shh* mRNA was detected at the center of retina with a shallow gradient but not at the periphery. At higher magnification, *Shh* mRNA expression was observed in a subset of cells in the ganglion cell layer (GCL), similar to that reported previously in various species, including mouse, chick, zebrafish, and *Xenopus* (Wallace and Raff, 1999; Neumann and Nuesslein-Volhard, 2000; Zhang and Yang, 2001; Perron et al., 2003) (Fig. 2*B*).

With the progression of retinal ganglion cell development, the *Shh* mRNA expression domain moves toward the periphery. At E9, the expression of *Shh* mRNA could only be detected at the retinal periphery, no longer in the center (Fig. 2*B*). At E12 and E15, no *Shh* expression was observed inside the retina (data not shown). Therefore, the expression domain of *Shh* appears to move with the differentiation wave. Because all RGC cells, including those at the periphery, have differentiated and their axons have reached the optic disc

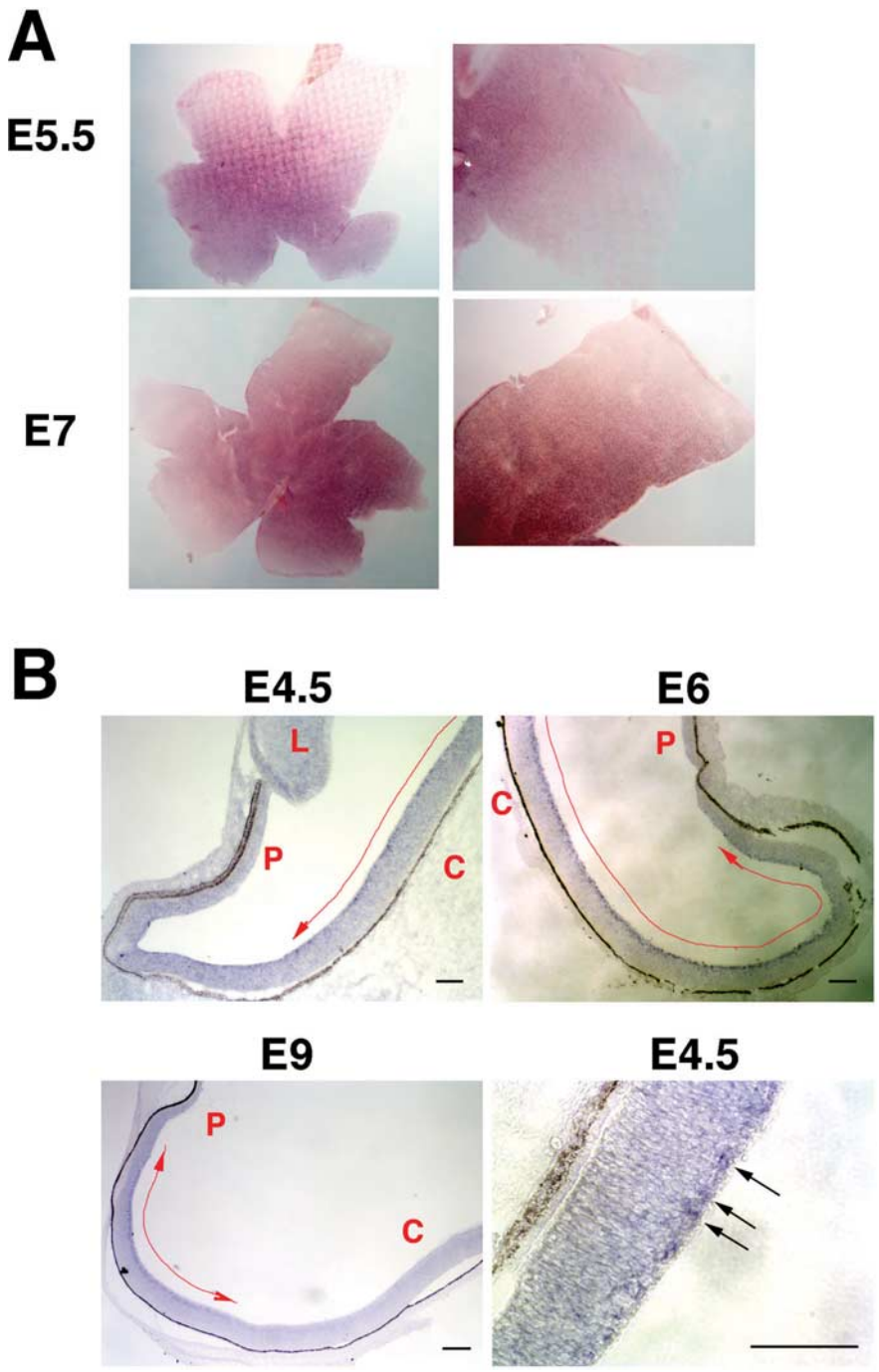

Figure 2. Expression of *Shh* mRNA in the chick retina. Retinas were flat mounted (***A***) or sectioned (***B***) and processed for *in situ* hybridization with a DIG-labeled *Shh* probe. ***A***, E5.5 and E7 retinas were shown on the ganglion side. Note that *Shh* is expressed in the center and not the periphery of the retina at E5.5 and E7. ***B***, On retinal cross sections, the expression of *Shh* mRNA was detected in a subset of cells in the GCL (black arrows). *Shh* mRNA is expressed in the GCL in the center of the retina at early stages such as E4.5. Gradually, the expression domain of *Shh* (marked with red arrows) moves toward the periphery of the retina. At E6, *Shh* expression is still higher in the center than periphery. At E9, the *Shh* mRNA expression is concentrated at the retinal periphery, and the expression in the center is diminished. L, Lens; P, periphery; C, center. Scale bars, 400 μm.

by E9, the expression domain of *Shh* appears to be trailing the differentiation wave at a relatively central position.

Results of gel culture experiments show that Shh acts as a positive factor to RGC axonal growth at low concentrations but as a negative factor at high concentrations

As the temporal shift of the positive activity from retinal center to periphery coincides well with the migration of the *Shh* expression domain, we tested whether Shh contributes to the detected positive activity. A chemical compound, cyclopamine, has been shown to inhibit Shh function highly specifically by binding to its coreceptor Smoothened (Incardona et al., 1998; Chen et al., 2002; Frank-Kamenetsky et al., 2002). Administration of cyclopamine to zebrafish, chicken, and mouse phenocopies the loss-of-function mutation of Shh (Dunn et al., 1995; Incardona et al., 1998; Berman et al., 2002; Chen et al., 2002; Charron et al., 2003). We included cyclopamine in the culture media and repeated the coculture gel culture experiments. As shown in Figure 1*G*, the positive activity detected in E5, E6 central, and E8 peripheral retinas was completely abolished by the addition of cyclopamine. These results suggest that Shh contributes to the endogenous positive activity to the RGC axonal growth inside the retina.

We next analyzed the effect of Shh on RGC axonal growth by using Shh-N in the gel culture assay. Because Shh is known to dictate different cell fates at different concentrations, we added Shh-N at a range of concentrations from 0.15 to 2.5 μg/ml to the culture media of retinal explants (Fig. 3). As the RGC axons grow out radially, we quantified the average length and number of axons per explant to determine the effect of Shh protein on RGC axonal growth. We compared all experimental data with the data from control experiments in which only control BSA was added to the culture media. Interestingly, we found that Shh had a dual effect on RGC axonal growth depending on its concentration (Fig. 3). At 0.15 μg/ml, Shh-N did not have a significant effect on RGC axons (axonal length, 172 $\pm$ 12.5 μm vs control, 164 $\pm$ 11.7 μm; $p > 0.1$; Student's t test). At 0.30 μg/ml, however, we observed a positive effect (195.7 $\pm$ 9.6 μm; $p < 0.001$), which peaked at 0.625 μg/ml (222.1 $\pm$ 20.8 μm; $p < 0.001$). Between 0.94 and 1.88 μg/ml, the effect of Shh-N was not significantly different from the control, with the p values between 0.05 and 0.1. However, at 2.5 μg/ml, Shh-N had a strong negative effect on RGC axonal growth (103.8 $\pm$ 9.4 μm; $p < 0.001$; Student's t test), similar to previously reported results (Trousse et al., 2001).

To confirm that the dual effects on axonal growth were attributable to the Shh-N protein and not impurities in protein preparations, we added cyclopamine with the Shh-N protein at the concentration of 0.625 or 2.5 μg/ml. Cyclopamine alone did not have a significant effect on RGC axons (155.9 $\pm$ 16.4 μm vs control, 164 $\pm$ 11.7 μm; $p > 0.05$). This is possibly attributable to the fact that the amount of Shh protein secreted by the tester explants themselves was very small, because the size of the ex-

plants was ~1/50 of the half retinas used for the coculture explant culture. However, coaddition of cyclopamine with Shh-N could effectively abolish both the positive and negative effects of Shh (143 ± 15.0 μm for 0.625 μg/ml Shh-N; 153 ± 20.9 μm for 2.5 μg/ml Shh-N, respectively), suggesting that these effects are specific to the Shh protein and its Smoothened receptor.

We also scored the number of axons that grew out from each explant (Table 1). Only those explants with similar sizes were scored. Possibly because of high variability within the data set, statistical analyses indicate that the number of axons that grew out per explant did not appear to differ significantly among different culture conditions. However, axons appeared to bundle more extensively in the 2.5 μg/ml Shh-N-added culture than in the BSA control and more extensively in the BSA control than in the 0.625 μg/ml Shh-N-added culture (data not shown).

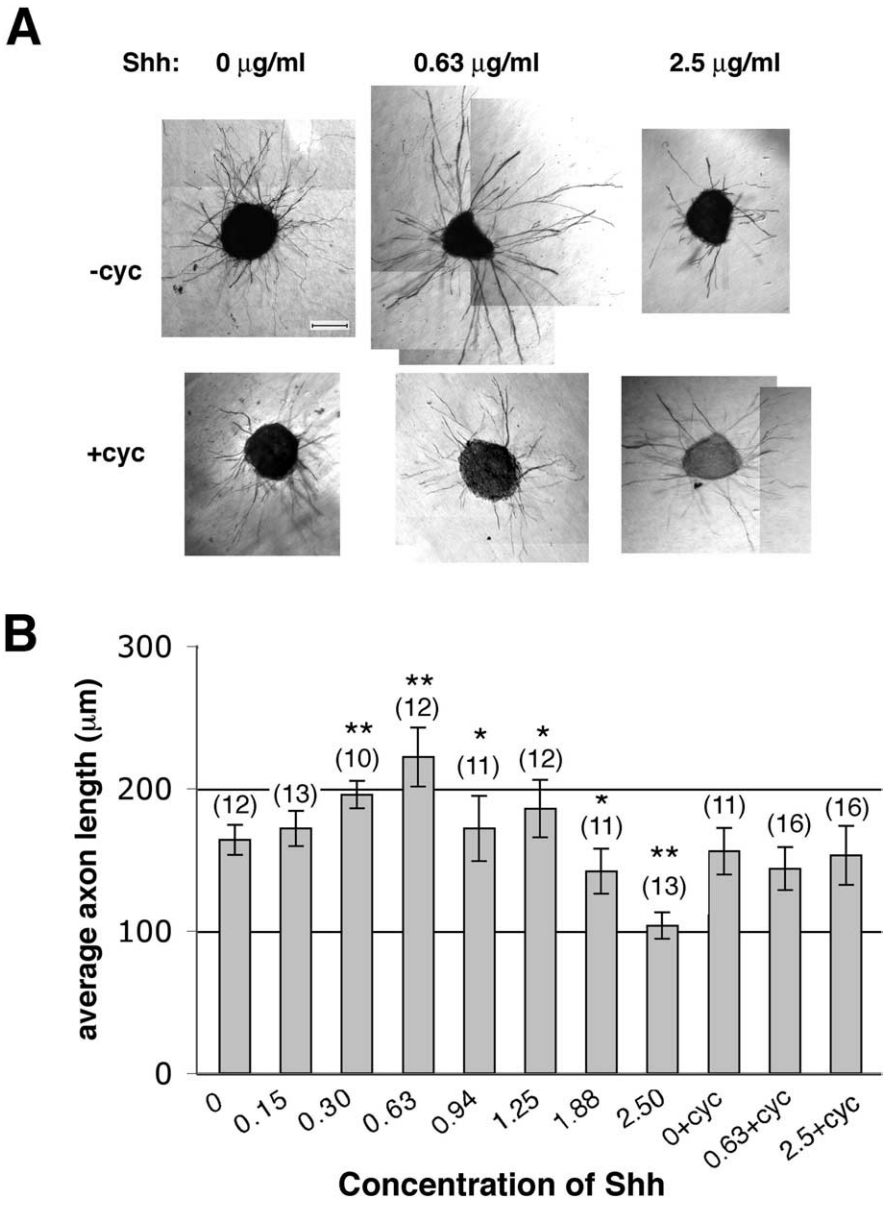

Figure 3. Shh acts as a positive factor at low concentrations and as a negative factor at high concentrations on RGC axons. Purified Shh-N protein was added to the gel culture at various concentrations with (+cyc) or without (−cyc) cyclopamine. Axons of retinal explants were stained, and some examples are shown in ***A***. Scale bar, 100 μm. The length of the axons was quantified, and all data were compared with the BSA-added control culture to determine whether a positive or a negative effect could be detected (***B***). The number of samples assayed is shown in parentheses. Statistical significance between the Shh-added and the BSA-control samples was assessed by Student's *t* test. *p* values between 0.05 and 0.1 are indicated by *, and *p* values <0.001 are marked by **. Note that cyclopamine blocked both the positive and the negative effects of Shh.

RGC growth cones respond relatively rapidly to the low concentration of Shh

It has been suggested previously that Shh-N acts directly on RGC growth cones to inhibit axonal growth at high concentration, 2.5 μg/ml (Trousse et al., 2001). To further characterize the positive effect of Shh at low concentrations, we performed a time-lapse video microscopic study. The baseline growth of the RGC axons was recorded for 1 h at 15 min intervals before either the control BSA or 0.5 μg/ml Shh-N was added to the culture medium. After addition of proteins, the recording was resumed for an additional 3 h at 15 min intervals. As shown in Figure 4*A*, in the control BSA-added culture, we observed a range of axonal behavior: some grew out, some retracted, whereas some remained static within the first hour after protein addition. However, in the 0.5 μg/ml Shh-N-added culture, none of the axons retracted within the first hour after the protein was added. Many axons grew steadily, whereas some remained static.

To quantify the results, the positions of all visible axons in the recordings at all time points were determined by the coordinates (*x*, *y*) using NIH Image software, and the cumulative growth for each axon was calculated (see Materials and Methods). As shown in Figure 4*B*, there is a significant difference in the average cumulative growth rate of the axons between the control BSA- and Shh-added samples, within a 1 h time frame. Within 30 min of protein addition, the average growth of axons was 0.044 ± 0.016 units in control samples (n = 28, 3 independent experiments) versus 0.103 ± 0.010 units in Shh-N-added samples (n = 41, 4 independent experiments; $p <$ 0.05; Student's *t* test). Between 30 and 60 min, the average growth was 0.036 ± 0.012 units in control samples versus 0.086 ± 0.004

Table 1. The mean number of axons per explant in cultures with different concentrations of Shh-N protein

Concentration of Shh-N (μg/ml)	Mean number of axons per explant	SD
0.00	63.9	21.7
0.15	59.0	32.5
0.30	44.1	26.6
0.63	79.2	29.1
0.94	61.2	31.9
1.25	65.9	24.1
1.88	56.0	17.2
2.50	55.7	25.0

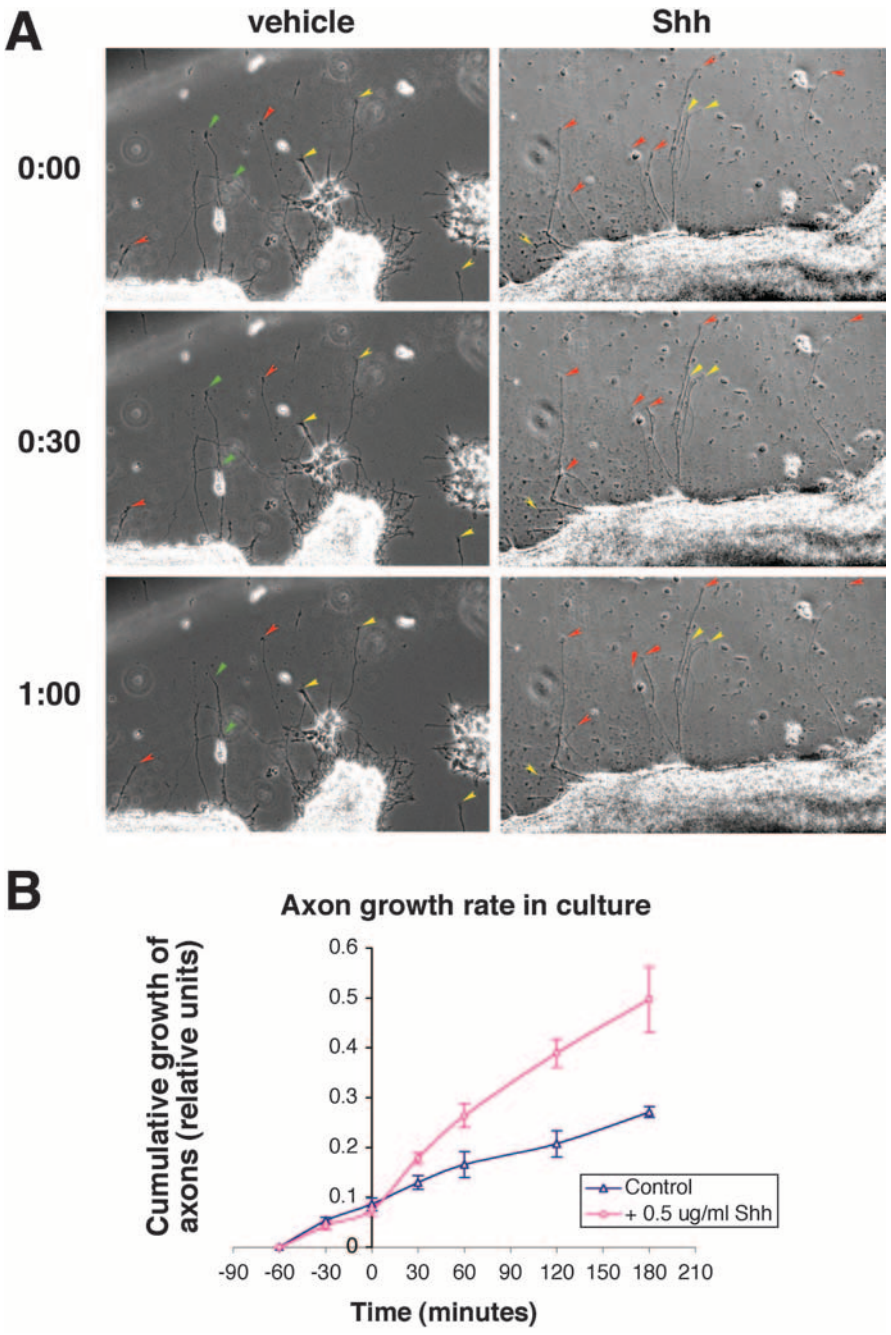

Figure 4. The response of RGC growth cones to low concentration of Shh is rapid. Time-lapse video microscopic studies were performed to record the growth of retinal axons during the addition of control BSA or 0.5 μg/ml Shh-N protein. ***A***, Photographs of the cultures were shown immediately (0:00), 30 min (0:30), and 1 h (1:00) after protein addition. Red, yellow, and green arrowheads mark the axons that have advanced, remained static, and retracted, respectively. ***B***, The growth rate of the axons in control BSA- or Shh-N-added cultures was shown by plotting average cumulative growth of the axons with time. A significant increase was observed in the average growth rate of the axons within 30 min after addition of 0.5 μg/ml Shh protein compared with that in the control BSA-added culture.

units in Shh-N-added samples ($p < 0.05$; Student's t test). The relatively rapid response to Shh protein suggests that the positive effect of Shh on RGC axonal growth is not likely through an indirect transcriptional regulation.

Results from stripe assay experiments show that Shh coated onto a solid surface can also influence the growth of the RGC axons in a concentration-dependent manner

We performed stripe assays by coating purified Shh-N protein onto glass coverslips into alternating first stripes at either a low concentration of 0.5 μg/ml or a high concentration of 2.5 μg/ml (see Materials and Methods). In some experiments, 4.0 μg/ml Shh protein was also used to ensure a good coating at high concentration. BSA protein was used to fill in between the stripes as the second stripes. Laminin was coated subsequently onto the entire surface to permit axonal growth. Control experiments were also performed with BSA proteins in both the first and second stripes.

As shown in Figure 5*A*, the axons grew equally well on the BSA-coated first and second stripes in the control experiments. We quantified the preference of the axons by calculating the ratio (n) of the axons present in the second versus the first stripes (total pixels on the second stripes over total pixels on the first stripes, normalized to the width of the stripes). From three independent experiments, the n value for the control experiment is 1.05 ± 0.04. When low concentration of Shh (0.5 μg/ml)-coated stripes were juxtaposing the BSA-coated stripes, the axons clearly preferred to grow on the 0.5 μg/ml Shh-coated stripes ($n = 0.09 \pm 0.02$; $p < 0.001$, three independent experiments; Student's t test). Conversely, RGC axons avoided the stripes coated with high concentrations (2.5 or 4.0 μg/ml) of Shh-coated stripes and preferentially grew on the BSA-coated stripes ($n = 14.48 \pm 2.01$; three independent experiments; $p < 0.05$; Student's t test). However, the number and length of the axons on the low concentration of Shh-coated stripes appeared similar to those on the BSA-coated stripes in the high-Shh concentration experiments (data not shown). These results suggest that Shh coated onto a solid surface can similarly influence the growth of the RGC axons in a concentration-dependent manner: a positive factor at a low concentration and a negative factor at a high concentration. Because RGC axons responded to the boundary between Shh- and BSA-coated stripes, the effect of Shh on the axons is more consistent with a direct effect rather than an indirect effect mediated by other proteins through transcriptional regulation by Shh.

Late injection of cyclopamine *in vivo* resulted in a severe loss of directed growth of the RGC axons inside the retina

To test whether the function of Shh is required for centrally directed RGC axon projection *in vivo*, we injected cyclopamine either into the optic vesicles at HH stage 10 (E1.5) or into the vitreal space next to the retina at E3.5. Cyclopamine at 1 mg/ml was mixed with HBC to increase solubility and stability of cyclopamine, as reported previously (Incardona et al., 1998). The injected retinas were analyzed by staining with the

anti-neurofilament antibody at E5.5 or E6. HBC alone was injected as a negative control.

In the samples injected with cyclopamine at the later stage E3.5, we detected abnormal RGC axon projection patterns in some areas (Fig. 6*B–D*). Because it is more difficult to target the vitreal space at E3.5 compared with optic vesicle injection at E1.5, the percentage of injected retinas that had the abnormal phenotypes was 42% ($n = 19$ retinas). A typical phenotype observed was the appearance of very disorganized axons that have completely lost directed projection. In many areas, no clear direction of axonal projection could be discerned (Fig. 6*B*,*C*). However, the distribution and fasciculation of the axons appeared essentially normal. We did not observe bare spots lacking axons or apparent overgrowth of axons. Another common phenotype observed was the abnormal crossing of axons in areas other than the optic disc. Because wild-type axons only converge at the optic disc, the abnormal crossings had an appearance of "ectopic optic discs" (Fig. 6*D*). These results suggest that blocking Shh function *in vivo* led to a severe loss of oriented axonal projection.

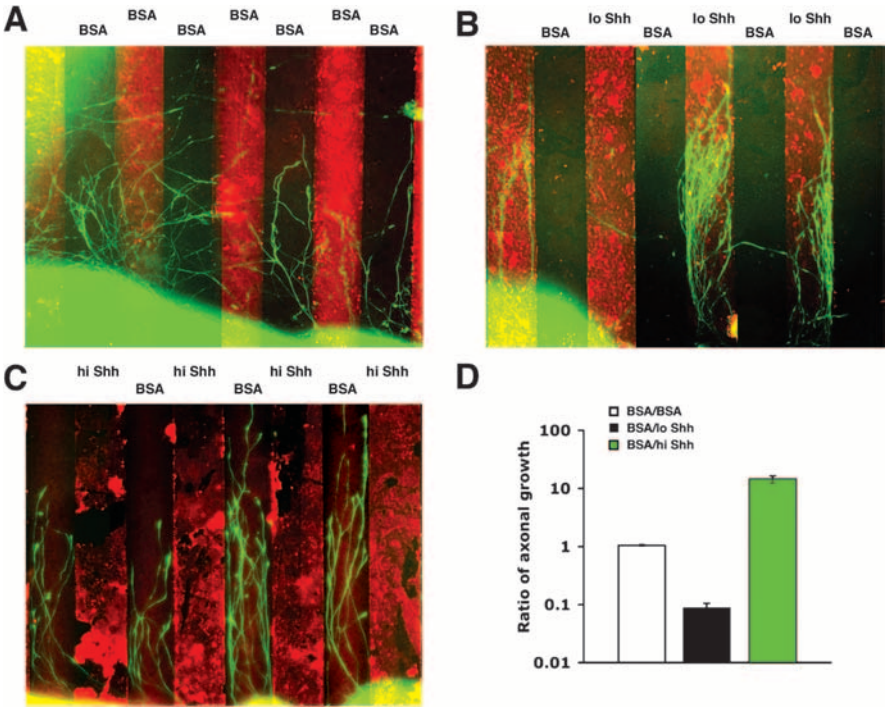

Figure 5. Shh directs the growth of RGC axons in a concentration-dependent manner. Purified Shh-N or BSA control proteins were coated onto glass coverslips in alternating stripes as the first stripes, which were marked by inclusion of a Cy3-conjugated fluorescent antibody. BSA protein was used to fill in as the second stripes. ***A***, Control experiment. RGC axons did not choose between the BSA-coated first and second stripes. ***B***, RGC axons preferred to grow on 0.5 μg/ml Shh-coated stripes over the BSA-coated stripes. ***C***, RGC axons chose to grow on BSA-coated and not high concentration (2.5 or 4.0 μg/ml) Shh-coated stripes. ***D***, Quantification of the stripe assay results.

However, in the samples injected with cyclopamine at an earlier stage, E1.5 ($n = 15$), the retinal axons appeared normal compared with the HBC-injected controls (Fig. 6*E*,*F*). The axons had a normal wavy "honeycomb-like" appearance typical of the axons at the early stage but were overall organized and centrally oriented. We also tried to inject cyclopamine twice, first into the optic vesicle at E1.5, followed by a second injection into the vitreal space at E3.5. The severity of the phenotype was similar to that of the single injection at E3.5 (39%; $n = 18$ retinas). Because the earliest RGC axon extension occurs at E3 inside the retina and the effect of cyclopamine is transient as a result of diffusion, the ineffectiveness of early injection at E1.5 suggests that Shh signaling may be important during rather than before axonal projection.

To determine whether blocking Shh function by cyclopamine had a gross effect on retinal differentiation, we stained the flat-mount retinas injected with cyclopamine at E3.5 with antibodies specific for various retinal cell markers. The expression of genes and proteins known to be expressed by the ganglion cells, Islet-1 and *Brn3a*, and by the amacrine cells, pax-6 and *Slit-1*, appeared normal (data not shown). The relatively mild phenotype on cell differentiation by cyclopamine may be a result of late injection and relatively brief presence of the drug attributable to diffusion in our experiments.

Misexpression of Shh by retrovirus resulted in severe disruption of retinal axon projection

To further determine the function of Shh in RGC axon projection *in vivo*, we took a gain-of-function approach to misexpress Shh protein by injecting a retrovirus, RCAS-Shh, into the optic vesicles. RCAS-Shh has been used successfully to express the full-length Shh protein in chick embryos (Riddle et al., 1993). Because mortality rate rose sharply after E7, we diluted the viral stocks to achieve partial infection and harvested retinas before E6.5. After flat mount, the retinas were double stained with an anti-viral capsid protein GAG antibody (P27; data not shown) and an anti-neurofilament antibody (270.7) to confirm viral infection and visualize axons, respectively.

In retinas injected with a control virus expressing a green fluorescent protein (GFP) protein, RCAS-GFP, the axons appeared normal as in uninjected wild type samples (Fig. 7*A*). In RCAS-Shh injected samples, however, the axons appeared abnormal in widespread areas (100%; $n = 24$ infected retinas), within or near the infected areas. The axonal phenotypes of Shh overexpression were remarkably similar to those observed in the cyclopamine-injected retinas at late stages (Fig. 7). As shown in Figure 7, *B* and *C*, a typical phenotype observed was the appearance of very disorganized axons that had completely lost directed projection. However, the distribution and fasciculation of the axons appeared essentially normal. Another common phenotype observed was the abnormal crossing of axons in areas other than the optic disc (39%; $n = 18$ retinas), resembling ectopic optic discs (Fig. 7*D–F*).

Despite grossly abnormal projection patterns, the axons were mostly confined within GCL, not misrouted into deeper layers of the retina, determined by adjusting the focal plane of the microscope (data not shown). This was further confirmed by analyzing the anti-neurofilament antibody staining on cross sections of the retinas injected with RCAS-Shh (Fig. 7*G–I*). The similar axonal phenotypes in the Shh overexpression and loss-of-function experiments suggest that the precise level of Shh protein expression inside the RGC layer is critical for the projection of RGC axons toward the optic disc.

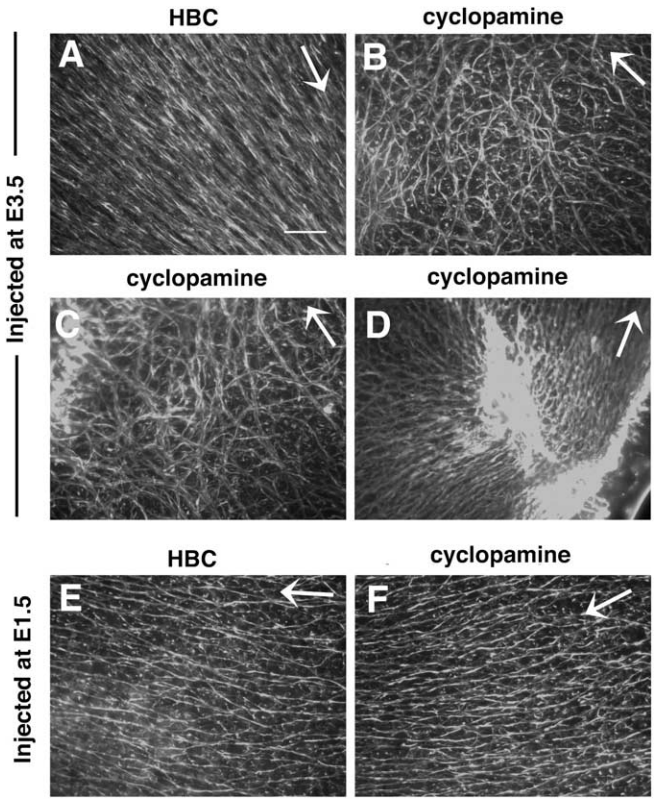

Figure 6. Inhibition of Shh function *in vivo* with injection of cyclopamine at E3.5 caused a severe abnormality in RGC axon projection. Cyclopamine/HBC (***B–D***, ***F***) or control HBC (***A***, ***E***) was injected into either the vitreal space next to the retina at E3.5 (***A–D***) or the optic vesicle at E1.5 (***E***, ***F***). The injected samples were harvested at E5.5–E6. Axons were visualized by staining with the anti-neurofilament antibody (270.7). ***B–D***, In some areas of the retinas injected with cyclopamine at E3.5, axonal projection appeared grossly disorganized and lacked central orientation toward the optic disc. In some areas, axons converged to form ectopic optic discs (***D***). ***E***, ***F***, Injection of cyclopamine at E1.5 did not alter the normal honeycomb appearance of the early axonal projections. Scale bar, 50 μm.

Discussion

In this paper, we show that Shh has a dual effect on the growth of the RGC axons, acting as a positive factor at low concentrations but as a negative factor at high concentrations. Furthermore, these effects may likely result from a direct interaction of Shh and the growth cones. *In vivo* experiments further demonstrate that the level of Shh protein expression is important for the projection of the RGC axons to the optic disc.

The following evidence support that Shh may play a major role in RGC axon projection inside the retina. First, results from *in vitro* gel culture experiments show that a secreted positive activity is present inside the retina, and localization of the endogenous positive activity changes from the center of the retina to the periphery, coinciding with the shift of the *Shh* expression domain. Moreover, the endogenous positive activity can be blocked by addition of cyclopamine, suggesting that the endogenous factor is a member of the Hedgehog (Hh) family. Cyclopamine has been shown to be highly specific for the Hh family (Incardona et al., 1998; Chen et al., 2002; Frank-Kamenetsky et al., 2002). The expression of the other two members of the Hh family, *Indian hedgehog* (*Ihh*) and *Desert hedgehog* (*Dhh*), is reported to be in the retinal pigment epithelium (Wallace and Raff, 1999; Perron et al., 2003). Therefore, the source of Hedgehog protein in the GCL is likely only Shh. Second, *Shh* is expressed in a pattern suitable for such a role. *Shh* is expressed in the ganglion cell layer in a shallow gradient high at the retinal center during the early axonogenesis period. The expression domain moves toward the periphery of the retina when the ganglion cells at the periphery start to send out axons. The dynamic expression pattern of the *Shh* gene provides a solution to the potential problem that ganglion cells at the periphery are far away from the optic disc (over 2 mm distance in chick). It would be difficult to achieve such long-range signaling if the guidance cues are only localized at the optic disc. Third, a change in Shh protein expression by either gain-of-function or loss-of-function approaches resulted in severe disruption in RGC axon targeting to the optic disc, suggesting that a precise regulation of Shh protein expression is critical. Fourth, *in vitro* experiments with gel culture, stripe assays, and time-lapse video microscopy demonstrate that purified Shh protein can affect the growth of the RGC axons in a concentration-dependent manner.

Shh has been shown previously to act directly on the growth cones of retinal and commissural axons (Trousse et al., 2001; Charron et al., 2003). It has been shown to act as an attractant on commissural axons but as a negative factor on RGC axonal growth. In both studies, a relatively high concentration of purified Shh protein (2.0 and 2.5 μg/ml, respectively) was used in various assays. We confirmed a previous observation that Shh at 2.5 μg/ml acts as a negative factor to RGC axonal growth and extended the observation to lower concentrations of Shh. Interestingly, the effect of Shh at low concentrations reverts to a positive effect. *In situ* hybridization results (Fig. 2*B*) have shown that the expression of *Shh* mRNA is still in the peripheral retina at E9 when all retinal cells, including those at the very periphery, have differentiated and extended axons to the optic disc. This shows that the *Shh* expression domain, although migrating with the differentiation wave, is at a relatively central position to the newly differentiated RGC neurons, suggesting that Shh likely acts as a positive factor *in vivo*. Based on the time required for the development of *in situ* hybridization reactions, *Shh* mRNA expression appears to be at a substantially lower level in the GCL inside the retina compared with notochord or floor plate (our unpublished observations). However, this does not exclude the possibility that a band of Shh protein at a relatively high concentration may be present because of protein accumulation. Currently, we do not have a way to estimate the amount of Shh protein *in vivo* because no suitable antibody is available. Because a multitude of factors are present *in vivo*, it is difficult to predict what constitutes a high or low concentration *in vivo* and whether any additional factor is required for Shh to act positively in the native environment (Song and Poo, 2001; van Horck et al., 2004).

Shh protein has been shown to elicit a positive turning response to the *Xenopus* spinal axon (Charron et al., 2003), suggesting that it can act as a guidance factor. Because the turning assay has not been used much on axons other than the *Xenopus* spinal axons, we do not know if it would be feasible to use the turning assay on chick RGC axons. Because many molecules affecting axonal growth can also act as guidance cues, it is possible that Shh may act as a guidance factor in RGC axon projection inside the retina. In our *in vivo* studies, we observed gross disorganization and loss of central orientation of axons but did not observe a significant change in axonal length or density inside the retina. These results appear more consistent with the role of Shh being a guidance factor rather than a growth regulator for the RGC axons.

Multiple factors are involved to ensure the proper guidance and target selection for RGC axons (Oster et al., 2004). As we have shown previously (Zhang et al., 2004), negative regulation may also be involved in addition to Shh to reinforce the central orien-

tation of the axonal projection. Once the axons are oriented to the center and extend to a certain length, we postulate that fasciculation may play a major role in stabilizing the orientation of axonal growth. Initially directed by the gradients of Shh and Zic3, the axons in close proximity to the optic disc exit the eyecup and are no longer influenced by the factors inside the retina. Through fasciculation with the central axons, the axons projected from more peripheral positions are also subsequently stabilized. Extensive fasciculation has in fact been observed after the initial axonal extension starting at E5.5. This may explain why the axons do not turn back when the expression domain of Shh moves to more peripheral positions to the axons.

Shh has been shown to play a role in retinal cell proliferation, differentiation, and patterning (Levine et al., 1997; Zhang and Yang, 2001; Dakubo et al., 2003). By *in vitro* gel culture, stripe assays, and time-lapse video microscopic studies, we have shown that Shh protein has effects on RGC axons, possibly independent of its role in other aspects of retinal development. The response of the growth cone to Shh protein is relatively quick (<30 min), which makes it unlikely to be an indirect response through transcriptional regulation or alteration of cell differentiation. In addition, the stripe assay results are not easily explainable by indirect effects of Shh. If Shh induces expression of secreted proteins, they would be distributed evenly in the medium and not necessarily be localized to the stripes. Because the axons clearly choose a stripe, it is unlikely that Shh induces a secreted protein that affects the axons; rather, it is Shh itself that directly acts on these growth cones. Moreover, some of the results from the *in vivo* experiments are also more consistent with Shh acting directly on the RGC axons. We only observed axonal phenotype by injection of cyclopamine at later stages, right at the beginning of axonogenesis. If the axonal phenotype resulted from the effect of Shh on neuronal differentiation, patterning, or regulation of downstream gene expression, early injection should have caused a more severe phenotype. However, definitive evidence would only come from experiments that define downstream elements of Shh involved in regulation of the growth of RGC axons. At present, we cannot rule out the possibility that the *in vivo* axonal phenotypes are partly attributable to the effect of Shh on patterning and cell differentiation.

Recently, ephrin-A2 has been shown to promote RGC axonal growth at low concentrations but inhibited growth at high concentrations (Hansen et al., 2004). However, soluble ephrin-A2 did not appear to have a detectable growth-promoting effect over a broad range of concentrations and oligomerization states, suggesting that the promoting effects may be attributable to direct receptor–ligand adhesion. Unlike ephrin-A2, we observed a similar concentration-dependent effect with both the soluble Shh protein and the Shh protein coated onto a solid surface, suggesting that the positive effect is unlikely attributable to adhesion. The concentration-dependent effect is possibly attributable to different intracellular signaling. Other morphogens such BMPs (bone morphogenetic proteins) and Wnt family proteins have also been shown to control axon guidance (Augsburger et al., 1999; Lyuksyutova et al., 2003; Yoshikawa et al., 2003). As a classical morphogen, Shh has been shown to induce discrete cell fates in a concentration-dependent manner (Ho and Scott, 2002; McMahon et al., 2003). Additional study is required to understand the mechanism underlying the concentration effect of Shh signaling involved in transcriptional regulation, as well as axon guidance.

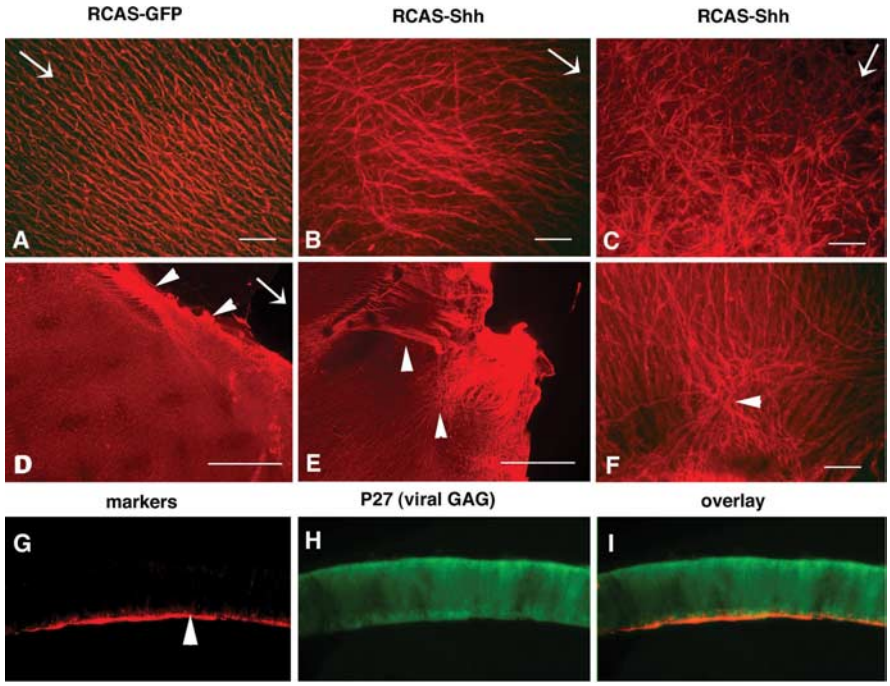

Figure 7. RGC axon projection toward the optic disc was similarly disrupted by misexpression of Shh. ***A***, Optic vesicles were injected with retroviruses expressing either Shh (***B***, ***C***, ***E***, ***F***) or a control GFP protein (***A***, ***D***) at HH stage 10–11. The infected retinas were harvested at E5.5–E6, flat mounted, and analyzed by staining with an anti-neurofilament antibody (***A–F***). As shown in ***A*** and ***D***, the RGC axons appeared normal in the control RCAS-GFP-injected retinas, similar to uninjected wild-type retinas (data not shown). However, in the RCAS-Shh-injected samples, the axons appeared grossly disorganized and lacked central orientation (***B***, ***C***). White arrows indicate the direction toward the optic disc. Another common phenotype caused by misexpression of Shh was the abnormal crossing of RGC axons, forming ectopic optic discs. Retinal axons normally converge only at the optic disc (arrowheads in ***D***) in which they exit the retina, as shown in this control RCAS-GFP-infected sample (***D***). However, misexpression of Shh resulted in abnormal crossing of RGC axons at ectopic sites before reaching the optic disc (***E***, ***F***, arrowheads). ***G–I***, RCAS-Shh-injected samples were sectioned and labeled with an anti-neurofilament antibody (***G***) and an anti-viral GAG antibody (***H***). Despite the gross mistargeting of RGC axons in the RCAS-Shh-injected retina, the axons were mostly confined to the GCL and were not present in the deeper layers of the retina. Scale bars: ***A–C***, ***F***, 50 μm; ***D***, ***E***, 400 μm.

References

Augsburger A, Schuchardt A, Hoskins S, Dodd J, Butler S (1999) BMPs as mediators of roof plate repulsion of commissural neurons. Neuron 24:127–141.

Bao ZZ, Bruneau BG, Seidman JG, Seidman CE, Cepko CL (1999) Regulation of chamber-specific gene expression in the developing heart by Irx4. Science 283:1161–1164.

Berman DM, Karhadkar SS, Hallahan AR, Pritchard JI, Eberhart CG, Watkins DN, Chen JK, Cooper MK, Taipale J, Olson JM, Beachy PA (2002) Medulloblastoma growth inhibition by hedgehog pathway blockade. Science 297:1559–1561.

Birgbauer E, Cowan CA, Sretavan DW, Henkemeyer M (2000) Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina. Development [Suppl] 127:1231–1241.

Brittis PA, Lemmon V, Rutishauser U, Silver J (1995) Unique changes of ganglion cell growth cone behavior following cell adhesion molecule per-

turbations: a time-lapse study of the living retina. Mol Cell Neurosci 6:433–449.
Charron F, Stein E, Jeong J, McMahon AP, Tessier-Lavigne M (2003) The morphogen Sonic Hedgehog is an axonal chemoattractant that collaborates with Netrin-1 in midline axon guidance. Cell 113:11–23.
Chen JK, Taipale J, Cooper MK, Beachy PA (2002) Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev 16:2743–2748.
Dakubo GD, Wang YP, Mazerolle C, Campsall K, McMahon AP, Wallace VA (2003) Retinal ganglion cell-derived sonic hedgehog signaling is required for optic disc and stalk neuroepithelial cell development. Development 130:2967–2980.
Deiner MS, Kennedy TE, Fazeli A, Serafini T, Tessier-Lavigne M, Sretavan DW (1997) Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. Neuron 19:575–589.
Dunn MK, Mercola M, Moore DD (1995) Cyclopamine, a steroidal alkaloid, disrupts development of cranial neural crest cells in *Xenopus*. Dev Dyn 202:255–270.
Flanagan JG, Vanderhaeghen P (1998) The ephrins and Eph receptors in neural development. Annu Rev Neurosci 21:309–345.
Frank-Kamenetsky M, Zhang XM, Bottega S, Guicherit O, Wichterle H, Dudek H, Bumcrot D, Wang FY, Jones S, Shulok J, Rubin LL, Porter JA (2002) Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. J Biol 1:10.
Giordano S, Laessing U, Ankerhold R, Lottspeich F, Stuermer CA (1997) Molecular characterization of E587 antigen: an axonal recognition molecule expressed in the goldfish central nervous system. J Comp Neurol 377:286–297.
Goldberg S, Coulombre AJ (1972) Topographical development of the ganglion cell fiber layer in the chick retina. A whole mount study. J Comp Neurol 146:507–518.
Halfter W, Schurer B (1998) Disruption of the pial basal lamina during early avian embryonic development inhibits histogenesis and axonal pathfinding in the optic tectum. J Comp Neurol 397:105–117.
Hamburger V, Hamilton HL (1992) A series of normal stages in the development of the chick embryo. 1951. Dev Dyn 195:231–272.
Hansen MJ, Dallal GE, Flanagan JG (2004) Retinal axon response to ephrin-as shows a graded, concentration-dependent transition from growth promotion to inhibition. Neuron 42:717–730.
Ho KS, Scott MP (2002) Sonic hedgehog in the nervous system: functions, modifications and mechanisms. Curr Opin Neurobiol 12:57–63.
Horder TJ, Mashkas A (1982) The developmental programme for retinal embryogenesis with special reference to the chick. Bibl Anat 23:103–123.
Incardona JP, Gaffield W, Kapur RP, Roelink H (1998) The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. Development [Suppl] 125:3553–3562.
Jin Z, Zhang J, Klar A, Chedotal A, Rao Y, Cepko CL, Bao ZZ (2003) Irx4 regulation of Slit1 expression contributes to the definition of early axonal paths inside the retina. Development 130:1037–1048.
Kahn AJ (1973) Ganglion cell formation in the chick neural retina. Brain Res 63:285–290.
Ledig MM, Haj F, Bixby JL, Stoker AW, Mueller BK (1999) The receptor tyrosine phosphatase CRYPalpha promotes intraretinal axon growth. J Cell Biol 147:375–388.
Levine EM, Roelink H, Turner J, Reh TA (1997) Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells *in vitro*. J Neurosci 17:6277–6288.
Lyuksyutova AI, Lu CC, Milanesio N, King LA, Guo N, Wang Y, Nathans J, Tessier-Lavigne M, Zou Y (2003) Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling. Science 302:1984–1988.
McMahon AP, Ingham PW, Tabin C (2003) Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol 53:1–114.
Neumann CJ, Nuesslein-Volhard C (2000) Patterning of the zebrafish retina by a wave of sonic hedgehog activity. Science 289:2137–2139.
O'Leary DD, Wilkinson DG (1999) Eph receptors and ephrins in neural development. Curr Opin Neurobiol 9:65–73.
Oster SF, Deiner M, Birgbauer E, Sretavan DW (2004) Ganglion cell axon pathfinding in the retina and optic nerve. Semin Cell Dev Biol 15:125–136.
Ott H, Bastmeyer M, Stuermer CA (1998) Neurolin, the goldfish homolog of DM-GRASP, is involved in retinal axon pathfinding to the optic disk. J Neurosci 18:3363–3372.
Perron M, Boy S, Amato MA, Viczian A, Koebernick K, Pieler T, Harris WA (2003) A novel function for Hedgehog signalling in retinal pigment epithelium differentiation. Development 130:1565–1577.
Rager U, Rager G, Frei B (1993) Central retinal area is not the site where ganglion cells are generated first. J Comp Neurol 334:529–544.
Richards LJ, Koester SE, Tuttle R, O'Leary DD (1997) Directed growth of early cortical axons is influenced by a chemoattractant released from an intermediate target. J Neurosci 17:2445–2458.
Riddle RD, Johnson RL, Laufer E, Tabin C (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 75:1401–1416.
Snow DM, Watanabe M, Letourneau PC, Silver J (1991) A chondroitin sulfate proteoglycan may influence the direction of retinal ganglion cell outgrowth. Development [Suppl] 113:1473–1485.
Song H, Poo MM (2001) The cell biology of neuronal navigation. Nat Cell Biol 3:E81–E88.
Tessier-Lavigne M, Goodman CS (1996) The molecular biology of axon guidance. Science 274:1123–1133.
Thanos S, Mey J (2001) Development of the visual system of the chick. II. Mechanisms of axonal guidance. Brain Res Brain Res Rev 35:205–245.
Trousse F, Marti E, Gruss P, Torres M, Bovolenta P (2001) Control of retinal ganglion cell axon growth: a new role for Sonic hedgehog. Development [Suppl] 128:3927–3936.
van Horck FP, Weinl C, Holt CE (2004) Retinal axon guidance: novel mechanisms for steering. Curr Opin Neurobiol 14:61–66.
Vielmetter J, Stolze B, Bonhoeffer F, Stuermer CA (1990) In vitro assay to test differential substrate affinities of growing axons and migratory cells. Exp Brain Res 81:283–287.
Wallace VA, Raff MC (1999) A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. Development 126:2901–2909.
Yoshikawa S, McKinnon RD, Kokel M, Thomas JB (2003) Wnt-mediated axon guidance via the *Drosophila* Derailed receptor. Nature 422:583–588.
Zhang J, Jin Z, Bao ZZ (2004) Disruption of gradient expression of Zic3 resulted in aberrant projection of the retinal ganglion axons to the optic disc. Development 131:1553–1562.
Zhang XM, Yang XJ (2001) Regulation of retinal ganglion cell production by Sonic hedgehog. Development [Suppl] 128:943–957.